IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of Daniel V Santi et al	:	Confirmation No. 9532
Application Serial No.: 10/820,975	:	Group Art Unit: 1633
Filed: April 7, 2004	:	
For: SYNTHETIC GENES	:	Examiner: Ileana Popa

X

Mail Stop Amendment

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

DECLARATION OF DR. SARAH J. REISINGER

Pursuant to 37 CFR §§ 1.131 AND 1.132

I, Sarah J Reisinger, declare as follows:

1. I am an inventor in the above-identified application.

2. On the face of the above-identified application, I am listed as Sarah J. Kodumal.

3. I affirm that the date of conception of the instant invention is prior to October 3, 2003, as demonstrated by the following evidence:

a) pages of a presentation, attached hereto as Exhibit 1, outlines the process for combining synthon fragments (i.e., the DNA segments and/or vector segments referred to in the claims) using rational restriction site assignments;

b) pages of project overview presentation, attached hereto as Exhibit 2, demonstrate conception of multiple synthon assembly and dual selection markers;

c) pages of my notebook, attached hereto as Exhibit 3, demonstrate conception of a dual-selection marker (tet/strep). The latter may be considered as a counterselectable marker;

d) a page of the progress report, attached hereto as Exhibit 4, demonstrates conception of a possibility to perform ligations "without purifying synthon fragments, and the procedure would only require plasmid purifications after each round of synthon insertion."

3. Additional experiments have been conducted through which actual reduction to practice of the invention have occurred. The pages from my notebook, attached hereto as Exhibit 5 describe experiments conducted in February 23-25, 2004. In these experiments, a successful four-piece ligation was performed using unpurified products of enzymatic digestion.

4. The aforementioned date of conception applies to claims 1-38 of the above-identified application, as well as to claim 39 as amended.

5. Due diligence in the instant invention was exercised by the Applicants from before October 3, 2003 through February 23-25, 2004, the date of actually reducing the invention to practice.

6. Exhibit 2 also shows unexpectedly superior results of using a two-marker vectors over one-marker vectors. Specifically, when we used one-marker vectors, 28% of the clones containing respective ligation products were false positives (i.e., did not contain the correct inserts in correct orientation). In contrast, when two-marker vectors were used (i.e., each and every clone tested had the correct insert in the correct orientation), the number of false positive clones was reduced to zero.

I, Sarah J Reisinger, hereby swear that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willfully false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued hereon.

Date: 12/1/08

Respectfully Submitted

Sarah J Reisinger

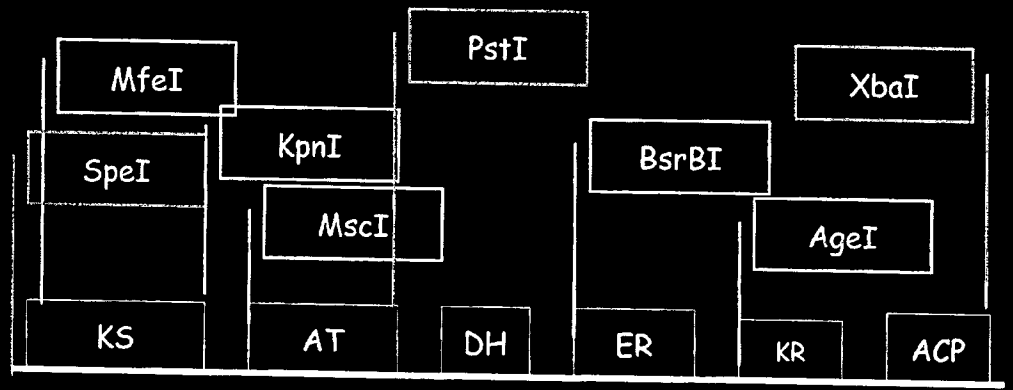
EXHIBIT 1

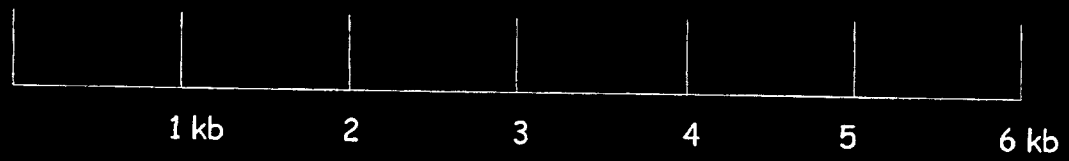
Module Gene Design

- Common restriction sites in all modules for modularity
  - flanking modules
  - flanking domains
- Restriction sites for sewing synthons together
- Intra-synthon restriction sites to correct errors in synthesis

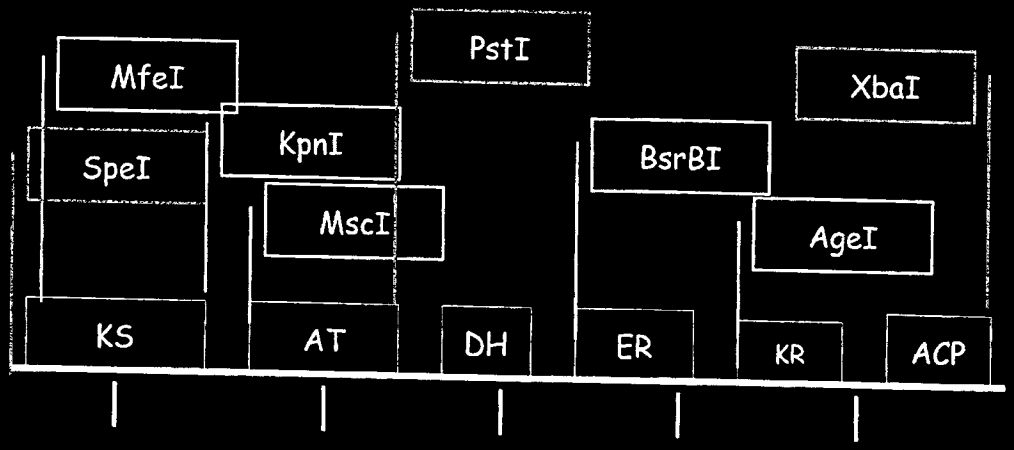
*Synthon = unit of DNA synthesized (350-750 bp);
Currently about 8-15 synthons per module

Module Gene Design

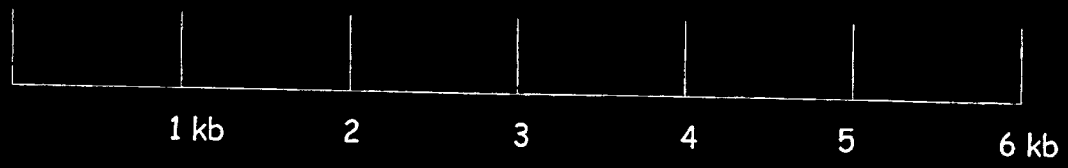




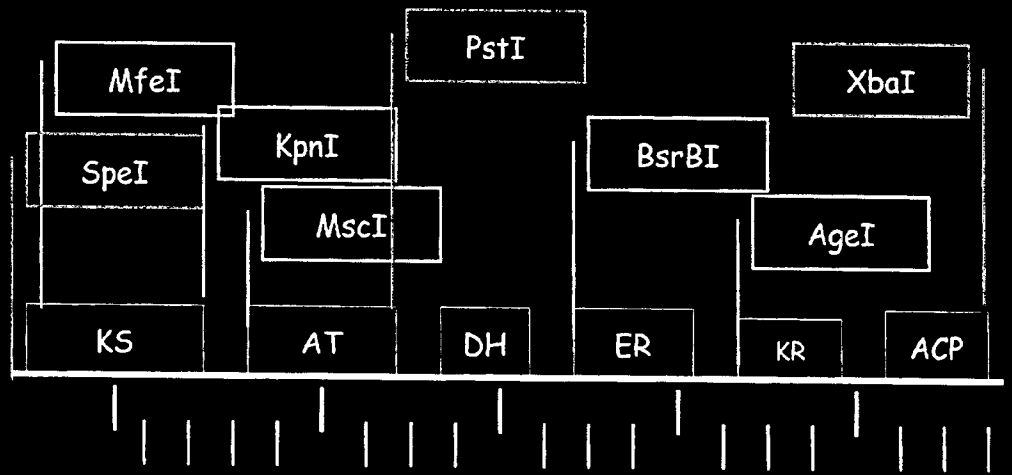
Module Gene Design



Additional sites for synthon edges



Module Gene Design



Additional intra-synthon sites for repair

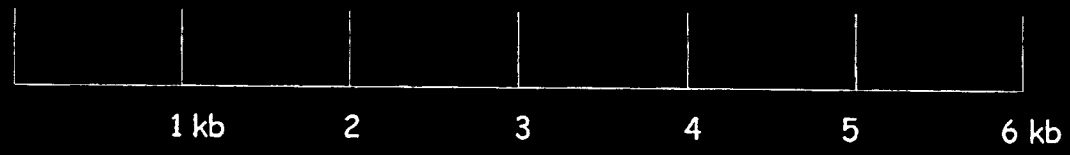


EXHIBIT 2

Gene Assembly ("Synthon Sewing")

Criteria:

Accurate

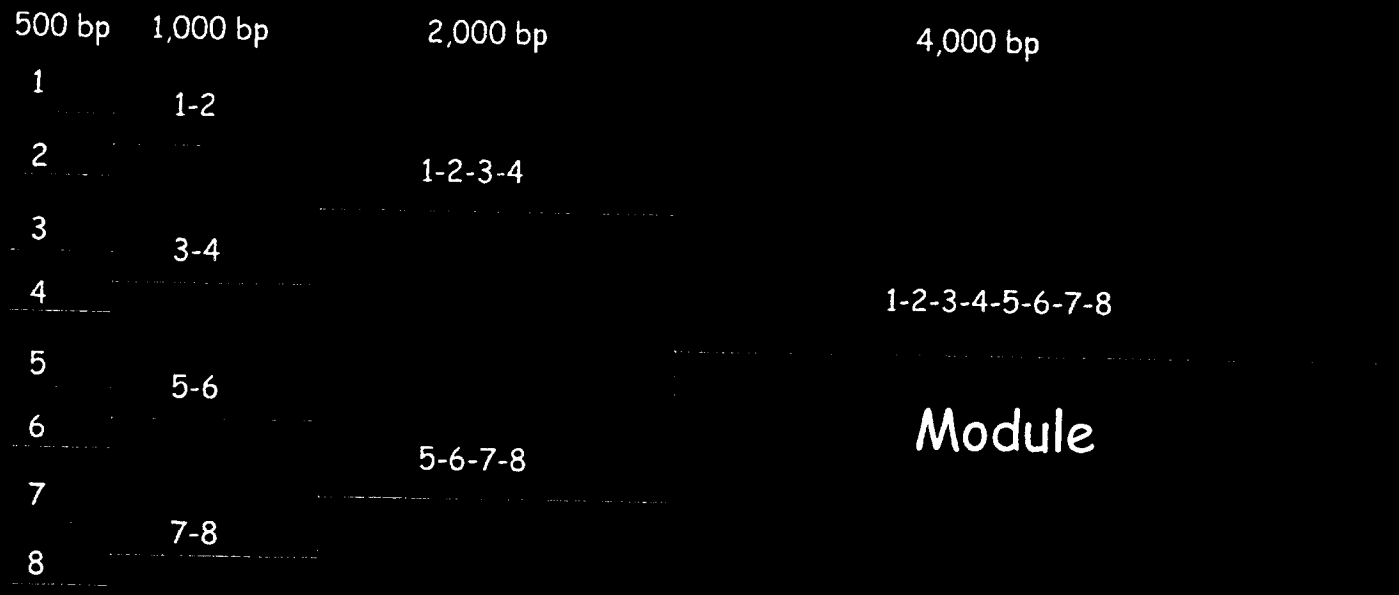
Amenable to HT

~10 plasmids containing
500 bp synthons

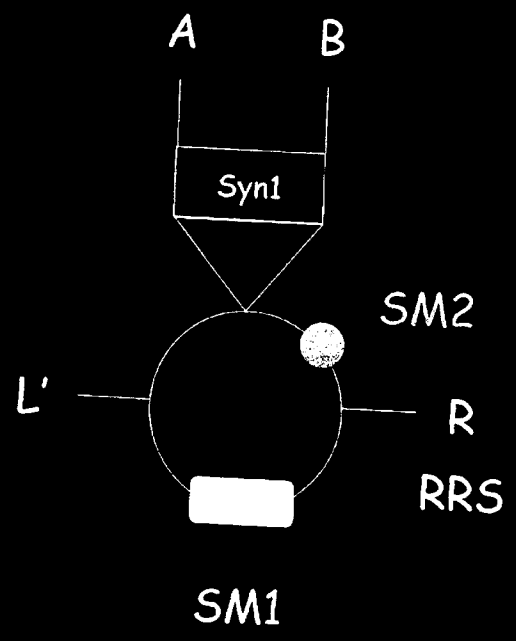
Automated stitching of synthons

5,000 bp module

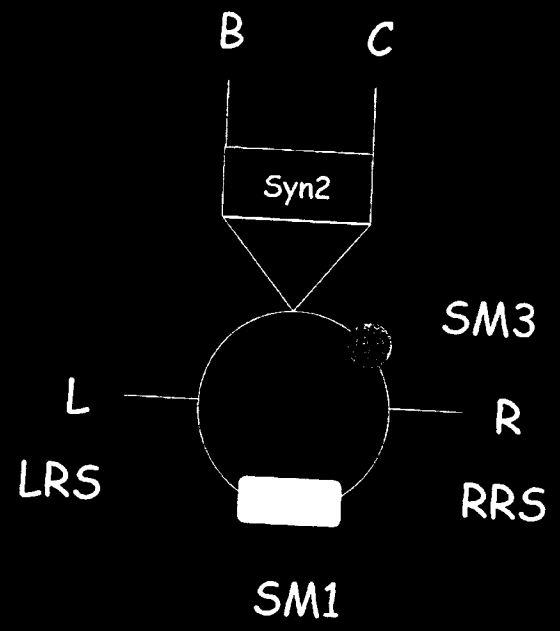
Assemble fragments into modules



Synthon Sewing



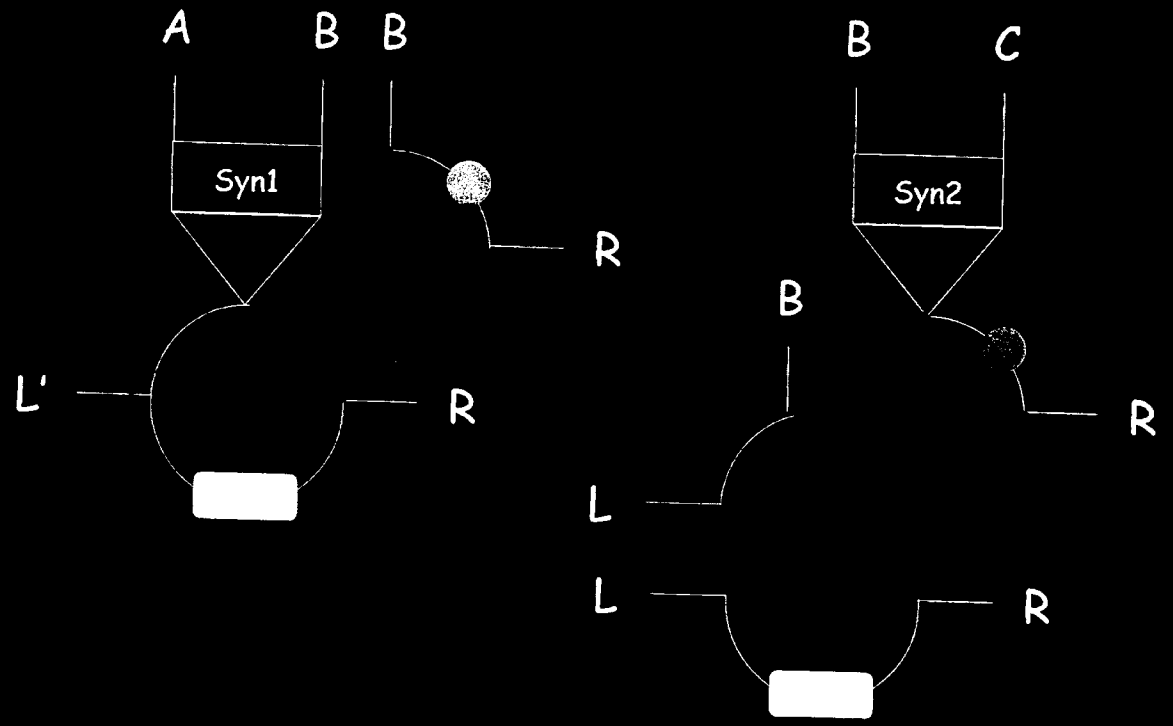
acceptor vector



donor vector

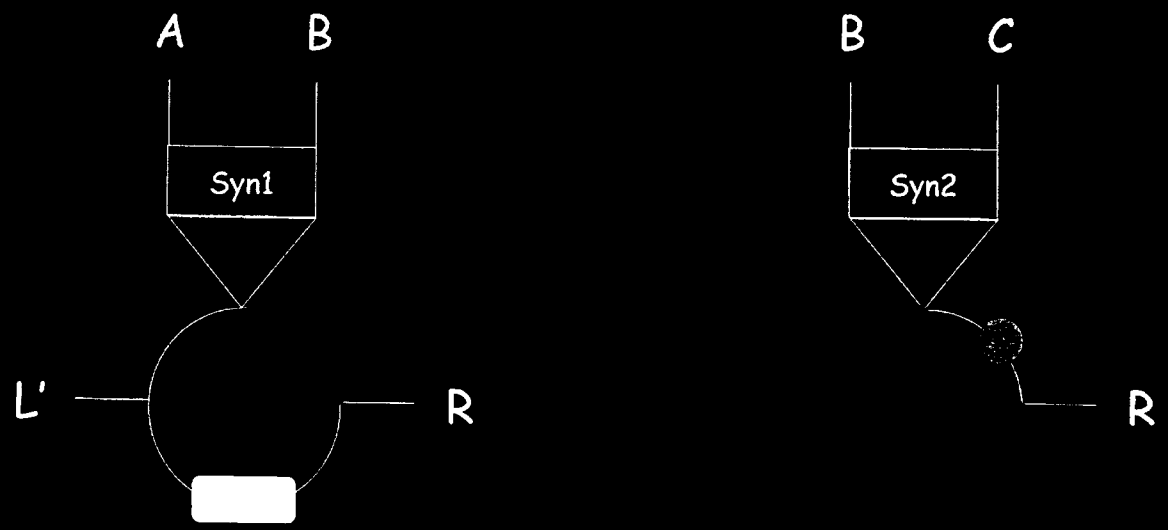
Synthon Sewing

Cut with B, R, L



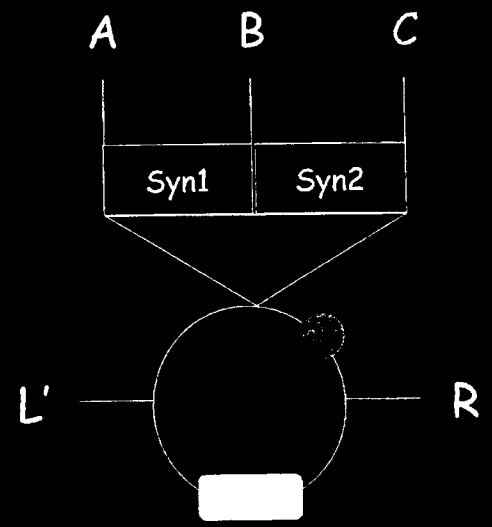
Synthon Sewing

Cut with B, R, L



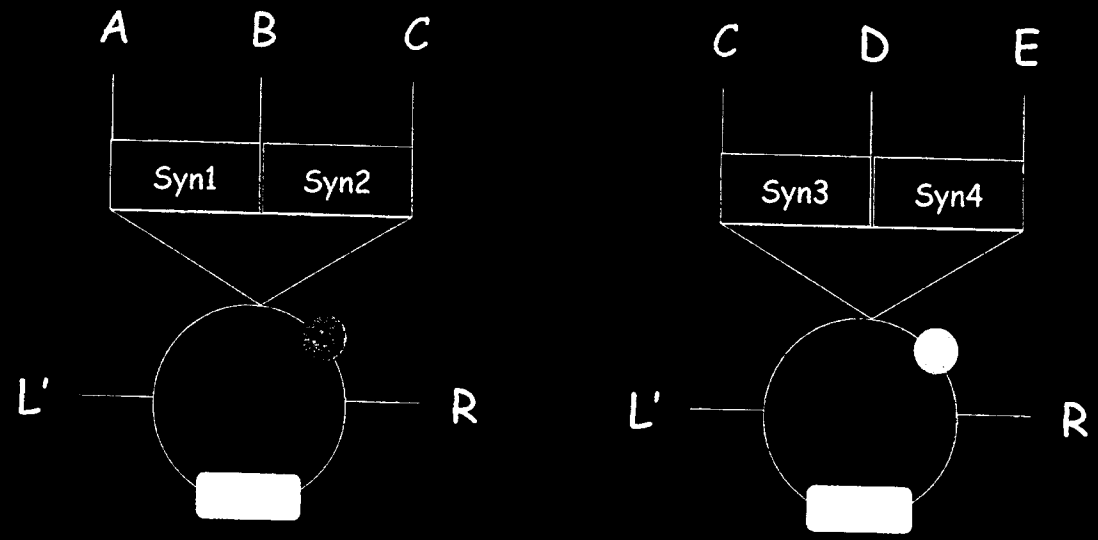
Synthon Sewing

Ligate, select, SM1 & 3

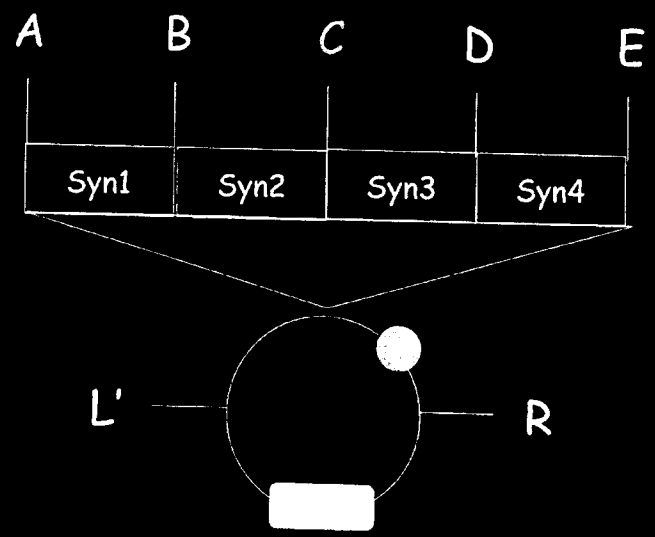


Synthon Sewing

Repeat processes with next mod pair



Synthon Sewing



Module Assembly (Synthon "Sewing") Results

- 18 complete modules constructed
- > 120 successful ligations
- 72% of clones screened were correct

Initial Synthon Sewing Limitations

- Method requires a number of different restriction enzymes (different one for each synthon edge)
  - Not very automatable
- Presence of false positive (background) clones

New Synthon Sewing Method

- Addition of second unique selectable marker on each plasmid to reduce false positives

- Utilize Type II restriction enzymes
  - Cuts DNA outside of recognition site

    5′ ... G G T C T C $(N)_1$^ ... 3′
    3′ ... C C A G A G $(N)_5$^ ... 5′

  - Use of different Type II enzymes that create compatible ends allows for cohesion of fragments

Results of Updated Synthon Sewing Method

- Selection scheme works extremely well
  - 100% correct in initial tests
  - Complete elimination of false positives
- Reduction in number of restriction enzymes allows for more genes to be assembled in parallel

EXHIBIT 3

From Page No. 121

I also designed PRIMERS for synthe cloning vector to amplify SM2-3 (neo & CAT)

Morphing Project
Synthon Cloning Vector

Introduction of Second Selectable marker

1. For Chloramphenicol (cat):
   -amplify using pACYC184
   -linearize template using Bam HI/ Xba I

Primers:

293-122-Cm Fwd
Forward (binds 384-365): 5'AATCCTGGTGTCCCTGTTGA 3'

293-122-Cm Rev
Reverse (binds 3751-3770): 5' CTCGAGTTATTCAGGCGTAGCACCAG 3'

2. For Kanamycin (neo):
   -amplify using pET26b
   -linearize with Xho I/Xba I

Primers:

293-122-KAN-Fwd
Forward (binds 3726-3745): 5' TGGTATCTGCGCTCTGCTGAAG 3'

293-122-KAN-Rev
Reverse (binds 4895-4876): 5' CTCGAGTTCAGGTGGCACTTTTCGGG 3'

RED is Xho I site (R) in cloning scheme

ORDER # **6104078**
CUSTOMER # **3002790**
P.O. # **10600**

Data Sheet

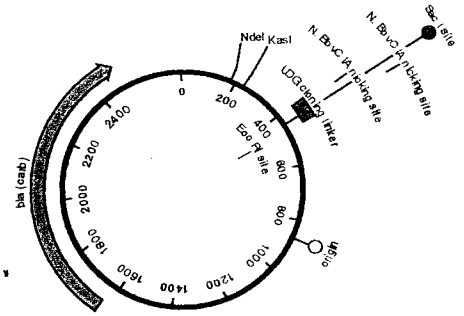
QIAGEN

Seq #	Seq Name	Seq 5' to 3'	OD	pmol	Len	MW	µg	E260	Tm	Scale	Purif.
5300	293-122-Cm_Fwd	AATCCTGGTGTCCCTGTTGA	16.14	88944.19	20	6098.98	542.47	181462.1	60.4	50 nmole	Salt Free
5301	293-122-Cm_Rev	CTCGAGTTATTCAGGCGTAGCACCAG	20.5	82357.37	26	7971.19	656.49	248915.2	67.75	50 nmole	Salt Free
5302	293-122-Kan_Fwd	TGGTATCTGCGCTCTGCTGAAG	18.03	89083.27	22	6757.4	601.97	202394.9	64.54	50 nmole	Salt Free
5303	293-122-Kan_Rev	CTCGAGTTCAGGTGGCACTTTTCGGG	21.34	89763.13	26	8009.2	718.93	237736.8	69.32	50 nmole	Salt Free

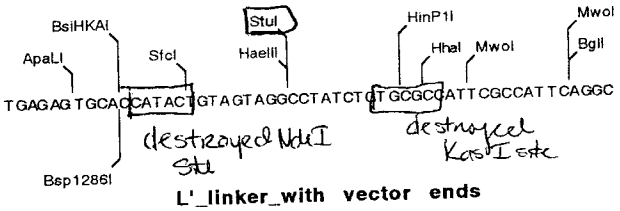
100pmol/µL

Synthon cloning vector: designed linker to destroy KasI site and insert L (StuI) or L' (EcoRV)

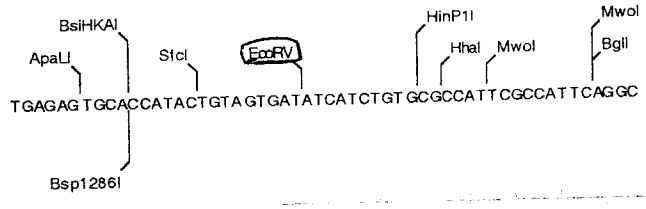
pKOS293-88-1_Synthon_vector



L_linker_with vector ends



L'_linker_with vector ends



Synthon Cloning Vector
Linker design for L and L'
Destroys NdeI and Kas I sites in process

(183-188 in MacVector File)
Nde I site
(235-240 in MacVector File)
Kas I site

5' C A T A T G N N N N N N N N N N N N N N G G C G C C 3'
3' G T A T A C N N N N N N N N N N N N N N C C G C G G 5'

Nde I /Kas I digest

5' C A
3' G T A T

G C G C C 3'
G 5'

LINKERS:

LINKER L SITE (Stu I)

5' P T A C T G T A G T A G G C C T A T C T G T 3'
3' G A C A T C A T C C G G A T A G A C A C G C G P 5'

A_StuI
B_StuI

LINKER L' SITE (Eco RV)

5' P T A C T G T A G T G A T A T C A T C T G T 3'
3' G A C A T C A C T A T A G T A G A C A C G C G P 5'

C_EcoRV
D_EcoRV

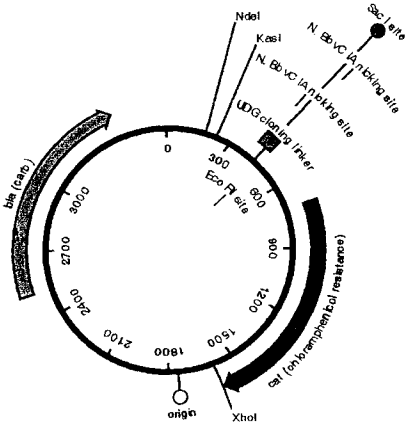
→ 293-125

From Page No 164

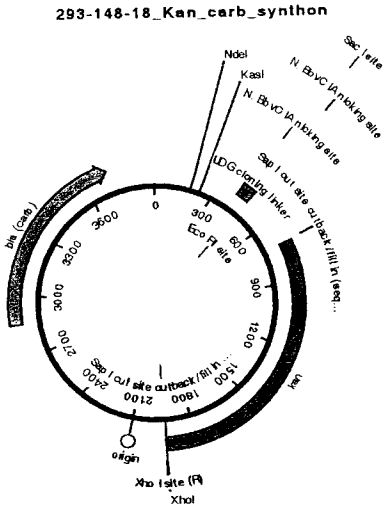
Also Redesigned oligos to insert L & L' linker.

Goal: Remove Nde I and Kas I sites from vector and insert linker containing either Stu I or Eco RV site. Stu I is blocked by overlapping dcm methylation, so no gg or cc near restriction sites. The diagrams of the vectors I am trying to insert linker into are shown, as well as a blow up of the region between Nde I and Kas I.

293-135-52_cm_carb_Synthon



293-148-18_Kan_carb_synthon



293-135-52_cm_carb_Synthon

NdeI KasI

GAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCC

200

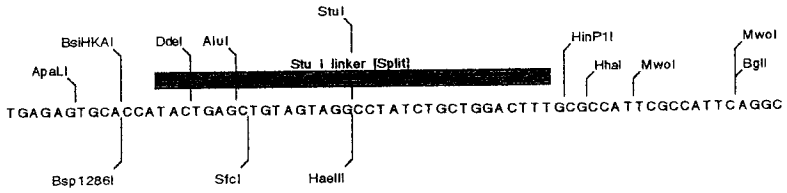
293-148-18_Kan_carb_synthon

NdeI KasI

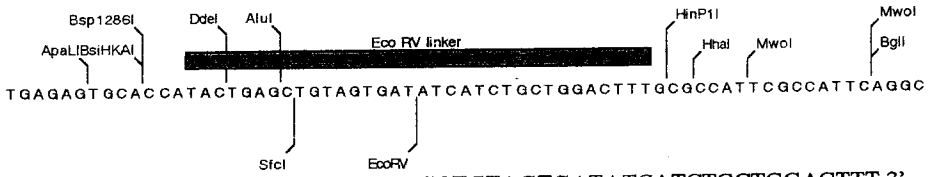
TGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCAT

200

L_linker_with vector ends



L'_linker_with vector ends



293-167-Eco RV 5'TACTGAGCTGTAGTGATATCATCTGCTGGACTTT 3'
293-167-Eco RV_RC 3' GACTCGACATCACTATAGTAGACGACCTGAAACGCG 5'

293-167- Stu I 5' TACTGAGCTGTAGTAGGCCTATCTGCTGGACTTT 3'
293-167- Stu I_RC 3' GACTCGACATCATCCGGATAGACGACCTGAAACGCG 5'

168 To Page No.

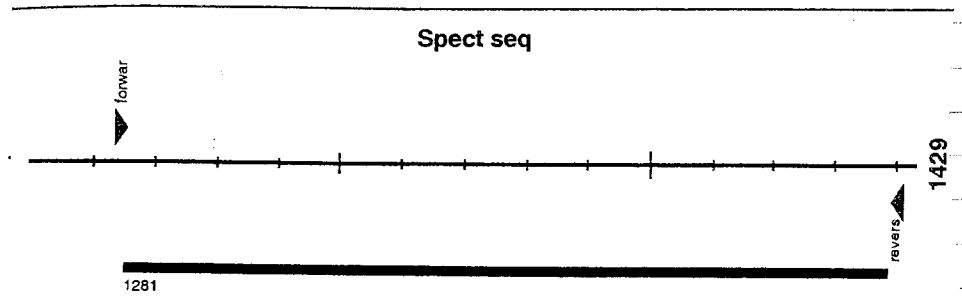
Oligos were designed to amplify tet a strep resistance
1) Blunt in PCR product at 5' ends
This will allow for 2 antibiotic markers in each plasmid for storage of synthesis-produced HTP stitch method with clear background.

eptomycin/spectinomycin Resistance Marker
uence Range: 1 to 1429

```
      10   20   30   40   50   60   70
ATCCTAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCA

      80   90  100  110  120  130  140
AGGCCGCCAATGCCTGACGATGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGC

     150  160  170  180  190  200  210
CTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCAG

     220  230  240  250  260  270  280
TGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAA

     290  300  310  320  330  340  350
CCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGG

     360  370  380  390  400  410  420
GGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGG

     430  440  450  460  470  480  490
AGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAAACATCATGAGGGAAGCGGTGA

     500  510  520  530  540  550  560
TCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCT

     570  580  590  600  610  620  630
GGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTT

     640  650  660  670  680  690  700
ACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAACTTCGGCTT

     710  720  730  740  750  760  770
CCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTG

     780  790  800  810  820  830  840
GCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTC
                                   BfuA I site

     850  860  870  880  890  900  910
GAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGG

     920  930  940  950  960  970  980
TAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAAC

     990 1000 1010 1020 1030 1040 1050
CTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGC

    1060 1070 1080 1090 1100 1110 1120
ATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGCC

    1130 1140 1150 1160 1170 1180 1190
TGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAAGAAGATCGCTT

    1200 1210 1220 1230 1240 1250 1260
GGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGC

    1270 1280 1290 1300 1310 1320 1330
AAATAATGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGATGCA

    1340 1350 1360 1370 1380 1390 1400
CTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTATTATTTTTAAGCGTGCATAA

    1410 1420
TAAGCCCTACACAAATTGGGAGATATATC
```

Project No.
Book No. 399 TITLE pUC19 LIC Vector improvement

From Page No. 3



imers for strep/spec. resistance

sulting fragment is 1281 bp in length

gtagggcttattatgcacgct reverse Strep_Rev
cagaaatgcctcgacttcgct forward2 Strep_For

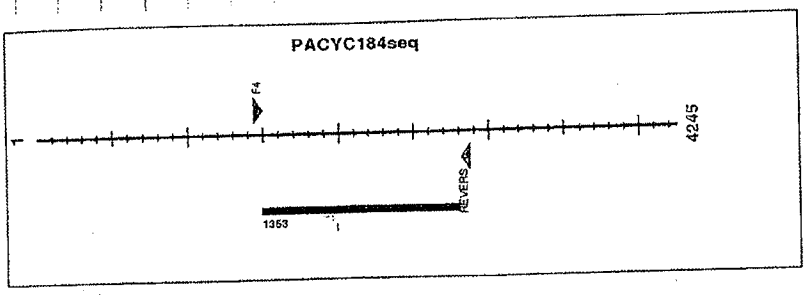
Use pKOS173-176 as template

1) Blunt in PCR fragment at EcoRV site. EcoRV site present in tet gene so cannot use both
2) Need to eliminate BfuAI site

Tet resistance marker

```
1480 1490 1500 1510 1520 1530 1540
CAGCCCCATACGATATAAGTTGTAATTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAG

                >Tet_resistance_marker
1550 1560 1570 1580 1590 1600 1610
TTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATC

1620 1630 1640 1650 1660 1670 1680
CTCGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGCGGG

1690 1700 1710 1720 1730 1740 1750
ATATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTGCTAGCGCTATATGCGTTGATGCAATT

1760 1770 1780 1790 1800 1810 1820
TCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTA

1830 1840 1850 1860 1870 1880 1890
CTTGGAGCCACTATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTCTACGCCGGACGCA

1900 1910 1920 1930 1940 1950 1960
TCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGA

1970 1980 1990 2000 2010 2020 2030
AGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCC

2040 2050 2060 2070 2080 2090 2100
GGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACC

2110 2120 2130 2140 2150 2160 2170
TACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTT

2180 2190 2200 2210 2220 2230 2240
CAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTCGCCGCACTTATGACTGTCTTCTTT

2250 2260 2270 2280 2290 2300 2310
ATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGGACCGCTTTCGCTGGA

2320 2330 2340 2350 2360 2370 2380
GCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCACGCCCTCGCTCAAGCCTTCGTCAC

2390 2400 2410 2420 2430 2440 2450
TGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGC

2460 2470 2480 2490 2500 2510 2520
TACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTTCTCGCTTCCGGCG

                                        Bfu A I site
2530 2540 2550 2560 2570 2580 2590
GCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCA

2600 2610 2620 2630 2640 2650 2660
AGGATCGCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCC

2670 2680 2690 2700 2710 2720 2730
GCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCG

2740 2750 2760 2770 2780 2790 2800
CGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGA

2810 2820 2830 2840 2850 2860 2870
TTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGC

2880 2890 2900 2910 2920 2930 2940
AGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGGTCCT
```

From Page No.



PRIMERS for Tet resistance:
Resulting fragment is 1353 bp

GCTCCAATTCTTGGAGTGGT REVERSE (Tet_Rev)
GCCCCATACGATATAAGTTG F4 (Tet_For)

ORDER # 6179684
CUSTOMER # 90122
P.O. # 14509

Oligonucleotide Data Sheet

Seq #	Seq Name	Seq 5' to 3'	OD	pmol	Len	MW	µg	E260	Tm	Scale	Purif.
11502	Tet_For	GCCCCATACGATATAAGTTG	14.06	71525.12	20	6101.05	436.38	196574.3	58.35	50 nmole	Salt-Free
11503	Tet_R	GCTCCAATTCTTGGAGTGGT	10.76	58037.96	20	6139.08	356.3	185395.9	60.4	50 nmole	Salt-Free
11504	Strep_For	CCTCAGAAATGCCTCGACTTCGCT	13.71	63236.84	24	7248.77	458.39	216804	66.28	50 nmole	Salt-Free
11505	Strep_Rev	GTAGGGCTTATTATGCACGCT	11.78	59144.53	21	6452.29	381.62	199173.1	60.61	50 nmole	Salt-Free

200 pmol/µL stock
20 pmol/µL work

Set up PCR

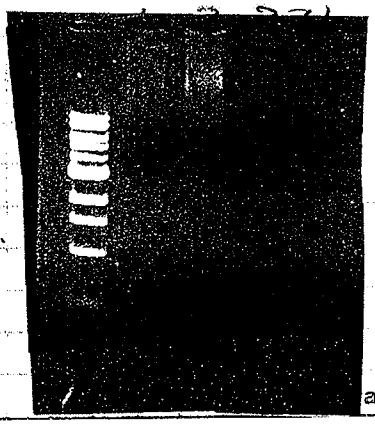
0.5 Vent polymerase
1 µL 10mM dNTPs
5 µL 10x PCR buffer
0.75 MgSO4
1 µL Probe (Tet or Strep 20pmol/µL)
1 µL template
40 µL water

PCR: 95 2m
25x [95 30sec
54 45sec
72 1mn30
72 10mn

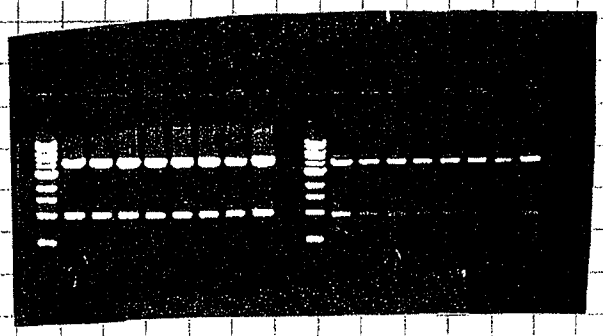
PCR did not work, but I could not see primer at all. Run out primers

Samples template

1 1:10 pBR322
2 1:20 "
3 1:10 pAM1109
4 1:20 "



om Page No. 16



For pKOS399-53 + strep = pKOS399-16-69

pKOS399-52 + tet = pKOS399-16-78

Site Directed Mutagenesis of 399-16-69 (strep/kan/carb)

Destroy BfuA I site in strep gene—design oligos for Quikchange (stratagene)

Changing GCAGGT (Ala Gly) to GCTGGT at position 899 in the vector

399-17-strep_sdm_for
5' GCGCAATGACATTCTTGCTGGTATCTTCGAGCCAGCC 3'

399-17-strep_sdm_rev
5' GGCTGGCTCGAAGATACCAGCAAGAATGTCATTGCGC3'

Site Directed Mutagenesis of 399-16-78 (tet/cm/carb)

Destroy BfuA I site in tet gene ---design oligos for Quikchange (stratagene)

Changing agGCAGGTa (Arg Gln Val) to agGCAAGTa at position 1285 in vector

399-17-tet-sdm_for
5' GCAGGCCATGCTGTCCAGGCAAGTAGATGACGACC 3'

399-17-tet-sdm_rev
5' GGTCGTCATCTACTTGCCTGGACAGCATGGCCTGC 3'

Cancel this Colony. BfuAI 15 min Good Luck

To Page No.

From Page No.

Primers for dU Cloning of Synthons:
Universal Amplification Primers

Currently we are using
316-4-For_Morph_dU:
5' GCUAUAUCGCUAUCGAUGAGCUGCCACTGAGCACCAACTACG 3'

316-4-Rev_Morph_dU:
5' GCUAGUGAUCGAUGCAUUGAGCUGGCACTTCGCTCACTACACC 3'

However, now that we are using the new type IIs restriction sites we want to add a better Need to have Forward have Bsa I site (5' GGTCTC3') and reverse to have Bbs I (5' GAAGAC3') site and a stuffer base (since cuts N2/N6)

So new primers to test will be:

Forward: 399-57-dU_For
5' GCUAUAUCGCUAUCGAUGAGCUGCCACTGAGCACAG**GGTCTC** 3'

*AG before Bsa I site was changed from CA to prevent methylation problems

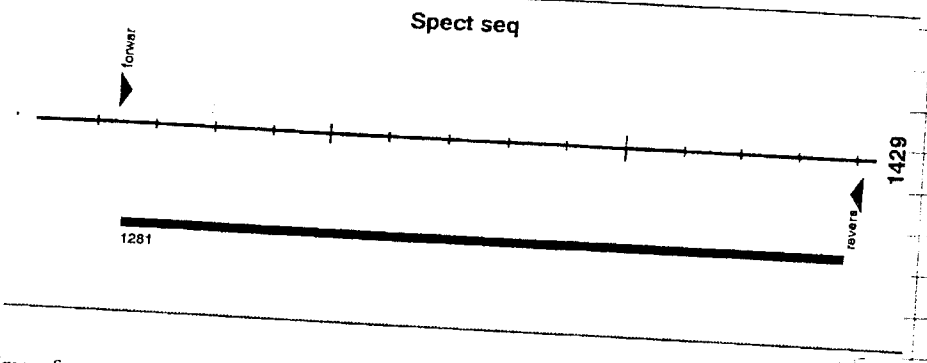
Reverse: 399-57-dU_Rev
5' GCUAGUGAUCGAUGCAUUGAGCUGGCACTTCGCTCAC**GAAGACC** 3'

*last C is stuffer base, but it cannot be a T, as it would cause methylation problems when Xba I site present in synthon.

Blue present in dU cloning vector.
RED is Modified T7 primer that is universal at 5' of all synthons
GREEN is modified T3 primer that is universal at 3' end of all sythons
PURPLE is the Bsa I site
ORANGE is the Bbs I site

To Page No.

EXHIBIT 4



imers for strep/spec. resistance

sulting fragment is 1281 bp in length

gtagggcttattatgcacgct reverse Strep_Rev

cagaaatgcctcgacttcgct forward2 Strep_for

Add Strep gene here

Use pKOS173-176 as template

1) Blunt-in 2nd Strep gene at Eco RV site. Eco RV site present in Tet^R gene so can inactivate Tet^R gene

2) Need to delete Bfu AI site

Tet resistance marker

```
1480 1490 1500 1510 1520 1530 1540
CAGCCCCATACGATATAAGTTGTAATTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAG
                    >Tet_resistance_marker
1550 1560 1570 1580 1590 1600 1610
TTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATC
1620 1630 1640 1650 1660 1670 1680
CTCGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGCGGG
1690 1700 1710 1720 1730 1740 1750
ATATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTGCTAGCGCTATATGCGTTGATGCAATT
1760 1770 1780 1790 1800 1810 1820
TCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTA
1830 1840 1850 1860 1870 1880 1890
CTTGGAGCCACTATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTCTACGCCGGACGCA
1900 1910 1920 1930 1940 1950 1960
TCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGA
1970 1980 1990 2000 2010 2020 2030
AGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCC
2040 2050 2060 2070 2080 2090 2100
GGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACC
2110 2120 2130 2140 2150 2160 2170
TACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTT
2180 2190 2200 2210 2220 2230 2240
CAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTCGCCGCACTTATGACTGTCTTCTTT
2250 2260 2270 2280 2290 2300 2310
ATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGGACCGCTTTCGCTGGA
2320 2330 2340 2350 2360 2370 2380
GCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCACGCCCTCGCTCAAGCCTTCGTCAC
2390 2400 2410 2420 2430 2440 2450
TGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGC
2460 2470 2480 2490 2500 2510 2520
TACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTTCTCGCTTCCGGCG
2530 2540 2550 2560 2570 2580 2590   Bfu A I site
GCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCA
2600 2610 2620 2630 2640 2650 2660
AGGATCGCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCC
2670 2680 2690 2700 2710 2720 2730
GCCTCGGCGAGCACATGGAACGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCG
2740 2750 2760 2770 2780 2790 2800
CGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGA
                    2840 2850 2860 2870
                    TGGAGCCAATCAATTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGC
                    2910 2920 2930 2940
                    CCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGGTCCT
```

Module Assembly From Synthons

Synthesis of each PKS module gene (~ 5,000 bp) requires the stitching together of about ten 500 bp synthons in a specific order. Conventionally this would require gel-purification of the individual synthons before ligation, and careful analysis of the product to make certain the new synthon has been introduced. Here, we describe an approach to circumvent this that utilizes a selectable marker to identify and discriminate the product from the original accepting vector. If successful, it should be possible to perform the ligations without purifying the synthon fragments, and the procedure would only require plasmid purifications after each round of synthon insertion.

The idea is to create two different vectors into which individual synthons (e. g. 1 through 12) are initially cloned (Figure 2). There are restriction sites (A, B, C, etc.) at both the left and right edges of the synthons (A-1-B, B-2-C, C-3-D, etc.). The two vectors would share a common selectable marker (e.g. SM1), and each would have a second distinct marker (SM2 or SM3) downstream of the synthon insert. Between the second marker and SM1 would be a "right" restriction site, R, and between SM1 and the left edge of the insert would a "left" restriction site L or L', different for each vector. Synthons 1, 4,6, 7 etc are be cloned into the vector with the SM2 marker, and 2,3,5,8 etc. would be cloned into the vector with the SM3 marker. The initial association of specific synthons (depending on their position in the module) with SM2 or 3 is important for downstream cloning steps.

The SM's in various vectors are as follows:

EXHIBIT 5

Project No. ______

Book No. 399 TITLE 4 Piece ligat

From Page to —

Testing 4 Piece ligation to see which

Samples for 4-piece ligation test with and without blunt cutter

1. Digest Samples

A.

6 uL of DNA 034-03 (Cm/Tet) #3 × 4
- 1 ul Bbs I
- 1 ul Xho I
- 3 ul 10 x BSA
- 3 ul buffer 2
- 16 water (to 30)

6 uL of DNA 034-04 (Kan/Strep) A1 × A2
- 1 ul Bbs I
- 1 ul Bsa I
- 3 ul 10 x BSA
- 3 ul buffer 2
- 16 water (to 30ul)

6 uL of DNA 034-02 (Cm/Tet) A5 × A6
- 1 ul Bbs I
- 1 ul Bsa I
- 3 ul 10 x BSA
- 3 ul buffer 2
- 16 water (to 30)

6 uL of DNA 034-07 (Kan/Stre in [illegible]
- 1 ul Bsa I
- 1 ul Xho I
- 3 ul 10 x BSA
- 3 ul buffer 3
- 16 water (to 30ul)

B.

6 uL of DNA 034-04
- 1 ul Bbs I
- 1 ul Xho I
- 3 ul 10 x BSA
- 3 ul buffer 2
- 16 water (to 30)

6 uL of DNA 034-05
- 1 ul Bbs I
- 1 ul Bsa I
- 3 ul 10 x BSA
- 1 ul Stu I
- 3 ul buffer 2
- 15 water (to 30ul)

6 uL of DNA 034-06
- 1 ul Bbs I
- 1 ul Bsa I
- 3 ul 10 x BSA
- 1 ul EcoRV
- 3 ul buffer 2
- 15 water (to 30)

6 uL of DNA 034-07
- 1 ul Bsa I
- 1 ul Xho I
- 3 ul 10 x BSA
- 3 ul buffer 3
- 15 water (to 30ul)

2. Verify digest on gel heat inactivate 20 min @ 65°C
3. Set up T4 o/n and quick ligations
4. Transform and select **Kan/Tet**

Set up T4 & quick ligation

A8 T4
- 3 uL 034-04 #3
- 2 uL 034-05 A2 (Bbs/Bsa)
- 3 uL 034-06 A6 (Bbs/Bsa)
- 2 ul 034-07
- 3 ul ligation buffer
- 17 ul [illegible]
- 1.5 l [illegible]

A8 Quick
- 3
- 2
- 3
- 2
- 10 [illegible]
- 1.5 ligase

To Page 139